

## Light-emitting diode with various sputum smear preparation techniques to diagnose tuberculosis

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### SUMMARY

**SETTING:** Mawenzi Regional Hospital, northern Tanzania.

**OBJECTIVE:** To determine the value of light-emitting diode (LED) microscopy in diagnosing tuberculosis (TB) on bleach-treated and direct sputum smears.

**DESIGN:** Sputum samples were collected from patients suspected of pulmonary TB who presented consecutively at the laboratory for smear evaluation between December 2009 and February 2010. Four smears were prepared from each specimen: conventional Ziehl-Neelsen (ZN), direct auramine, bleach centrifugation and bleach short sedimentation auramine smears. A light microscope was used to examine ZN smears and an LED fluorescent microscope to examine auramine-stained smears.

**RESULTS:** Of the 267 sputum samples examined, respectively 78 (29%), 62 (23%), 74 (28%) and 48 (18%)

were acid-fast bacilli (AFB) positive by the bleach centrifugation, bleach short sedimentation, direct auramine and ZN methods. Bleach centrifugation identified 30 (11%) more positives than ZN microscopy ( $P < 0.001$ ), but was not superior to the direct auramine method ( $P = 0.46$ ), which yielded 26 (10%) more positives than ZN microscopy ( $P < 0.001$ ). Fluorescent LED required a shorter smear reading time (1.5 min on average), while the light microscope took 4 min ( $P < 0.001$ ).

**CONCLUSION:** Fluorescent LED microscopy with direct smear preparation is rapid and effective. Further studies are needed to ascertain its performance under routine conditions.

**KEY WORDS:** bleach; microscopy; light-emitting diode; Ziehl-Neelsen

DIAGNOSING TUBERCULOSIS (TB) is still a major challenge in TB control programmes, as is reflected in the low global case detection rates, particularly in resource-poor settings.<sup>1</sup> Ziehl-Neelsen (ZN) smear microscopy remains the primary TB diagnostic tool, despite its poor sensitivity.<sup>2</sup> As the development and implementation of new techniques in resource-poor settings takes time,<sup>3</sup> improving the diagnostic performance of the available methods could increase case detection rates.

Novel approaches to improve the quality and suitability of smear microscopy are under evaluation.<sup>4</sup> Digestion of sputum with household bleach prior to smear preparation and concentrating the bacilli by either centrifugation or gravity sedimentation were found to improve its diagnostic yield.<sup>4–7</sup> In addition, studies have shown promising results with regard to improved smear reading using light-emitting diode (LED) microscopy.<sup>8</sup>

The present study aimed at determining the value of LED-based fluorescent microscopy (LED-FM) on

bleach treated and direct sputum smears compared to the conventional ZN method in the diagnosis of pulmonary TB (PTB).

### METHODS

#### *Design and study population*

This was a cross-sectional laboratory-based study. Between December 2009 and February 2010, all treatment-naïve PTB suspects aged  $\geq 15$  years who presented at the regional hospital of Tanzania's Kilimanjaro Region and who were referred to the laboratory for sputum smear evaluation were eligible.

The study protocol was approved by the Institutional Ethics Review Board of the Kilimanjaro Christian Medical Centre (KCMC). Participants provided written informed consent.

#### *Specimen collection and processing*

Patients who agreed to participate were instructed on how to produce and submit an adequate specimen.<sup>9</sup>

A minimum of 5 ml of sputum<sup>10</sup> was collected from each participant in a leak-proof, wide-mouthed plastic container with lid, and transported to the KCMC TB culture laboratory on the same day.

#### *Direct smears*

The yellowish, cheesy-mucus, purulent or blood-stained part of the specimen was smeared on two new labelled glass slides using a disposable plastic loop. The slides were air-dried and heat-fixed on an electric slide warmer (65–75°C) for 2 h.<sup>11</sup> One randomly chosen slide was stained using a ready-to-use hot ZN kit (BD, Franklin Lakes, NJ, USA) with a concentration of 0.3% carbol-fuchsin, 3% acid alcohol and 0.3% methylene blue. Stained smears for the detection of acid-fast bacilli (AFB) were read using an Olympus CX31 light microscope at 1000× magnification. The International Union Against Tuberculosis and Lung Disease (The Union) grading scale was used to interpret smear results.<sup>12</sup> The microscopic appearance of the smears was defined as 'good' if blue cellular elements were present, 'too thick' if cells/debris were lying on top of each other, 'too thin' in case of insufficient background, 'under-decolorised' if the background appeared purple/red and 'overheated' if red crystals were seen.<sup>13</sup>

The other slide was stained with a ready-to-use auramine kit (BD) with a concentration of 0.1% auramine-O, 0.5% acid alcohol and 0.1% potassium permanganate. The slide was examined at 400× magnification using an LED-based fluorescent microscope (LW Scientific, Lawrenceville, GA, USA) to detect yellow fluorescent rods on a dark background. The microscopic appearance of the smears were defined as 'good' if adequate dark background elements were present, 'too thick' if bulky smear material overwhelmed the fluorescent objects, 'too thin' in case of insufficient background and 'under-decolorised' if the background appeared fluorescent yellow throughout.

#### *Bleach digestion*

The remaining sputum sample was liquefied by adding an approximately equal volume of undiluted,<sup>7</sup> locally obtained 3.5% household bleach (Topex Bleach Regular; Supersleek Ltd, Nairobi, Kenya). To reduce the risk of oxidation, the household bleach was stored in a dark place where the temperature did not exceed 23°C. Moreover, as hypochlorite loses its chlorine content over time, bleach solution with less than 6 months from date of manufacture was used.<sup>14</sup>

The mixture was incubated for 15 min at room temperature with regular, gentle hand shaking. Half of the portion was then transferred to a 50 ml sterile disposable conic tube and spun at 3000× g for 15 min.<sup>5</sup> The remaining portion was kept at a 45° slant for 30–45 min (short sedimentation).<sup>15</sup>

A smear was prepared by putting two drops from

the sediment of centrifuged specimen on a glass slide. From the short sedimentation portion of the specimen, a smear was prepared by carefully taking two drops from the corner of the tube using a plastic Pasteur pipette. Smears were air-dried and heat-fixed on an electric slide warmer (65–75°C) for at least 2 h and fluorochrome-stained.<sup>12</sup> Slides were examined at 400× magnification using LED-FM to detect yellow fluorescent rods. The Union quantitation scale was used to interpret the smear results.<sup>12</sup> The microscopic appearance of the bleach-treated smears was documented as for direct auramine.

#### *Reproducibility considerations*

All bleach-treated and direct smears were prepared and stained by one of the investigators (MH) throughout the study period. The smears were read by two experienced laboratory technicians who were blinded to each other's results. Blinding was ensured by randomly re-assorting slides of a batch that had been evaluated by the first reader before it was given to the second reader. Results were documented on separate data capture forms for each method.

#### *Data management and analysis*

Data were analysed using SPSS version 15.0 for Windows (Statistical Package for the Social Sciences Inc, Chicago, IL, USA). As all smears were examined by two readers, the average yield was taken to establish the smear positivity rate for each method. The absolute percentage increment was calculated by taking the difference of any of the modified methods and conventional ZN microscopy divided by the total number of specimens. McNemar's  $\chi^2$  test was used to compare the results of duplicate smears. Cohen's kappa coefficient, with the corresponding 95% confidence interval (95%CI), was used to assess inter-reader agreement. Paired *t*-test was used to compare the average time required for smear reading. All statistical analyses were conducted with a significance level of 5%.

#### *Quality assurance/quality control*

Specimens were processed within 48 h of collection and evaluated on the same day of smear preparation. For each batch of staining reagent, positive (*Mycobacterium tuberculosis* H37RV) and negative (*Escherichia coli*) control smears were included. As part of internal quality control, 100% of positive and an arbitrary 5% of negative smears were re-examined on a weekly basis by an experienced laboratory technician who was blinded to the previously reported results. The original results were used for statistical analysis.

The KCMC TB culture laboratory is involved in an external quality assessment with the UK National External Quality Assurance Scheme (UK-NEQAS) for Microbiology. The assessment occurs every 3 months.

## RESULTS

During the study period, a total of 366 PTB suspected cases presented at the laboratory unit for sputum smear evaluation; 76 patients were not recruited: 13 were aged <15 years and 63 were already on anti-tuberculosis chemotherapy. Of the remaining 290 patients, who were asked to submit sputum, 23 specimens were regarded as inadequate in terms of quality and quantity. A final 267 sputum samples were therefore available for analysis. Of these, 78 (29%) were AFB-positive by bleach centrifugation, 62 (23%) by bleach short sedimentation and 74 (28%) by the direct auramine method, while conventional ZN microscopy identified 48 (18%) positive specimens. The average time ( $\pm$  standard deviation) required to examine a smear using LED-FM was 1.5 ( $\pm$  0.7) min and that of conventional ZN microscopy was 4.0 ( $\pm$  1.3) min ( $P < 0.001$ ).

### *Absolute per cent increment by the modified methods*

The absolute per cent increment gained by the three modified techniques is summarised in Table 1. The bleach centrifugation method identified 30 more positive smears (11% increment, 95%CI 7.3–15) than conventional ZN microscopy ( $P < 0.001$ ). The direct

auramine method detected an extra 26 positive smears, with an increment of 10% (95%CI 5.9–13.5) over conventional ZN microscopy ( $P < 0.001$ ). Compared to the direct auramine method, bleach centrifugation identified four more positives (1% increment), but the difference was not statistically significant ( $P = 0.48$ ). Bleach short sedimentation detected 14 extra positives, with an increment of 5% (95%CI 0.5–8.9) over conventional ZN ( $P = 0.02$ ).

### *Quantitation scale for acid-fast bacilli*

The quantitation results are summarised in Table 2. A total of 30 smears were identified as 'scanty or 1+' positive by the bleach centrifugation method. Of these, 25 (83%) were reported as 'negative' by conventional ZN ( $\chi^2 = 30.2$ ,  $P < 0.001$ ). Similarly, the direct auramine method identified 26 smears as 'scanty or 1+' positive, of which 22 (85%) were reported as 'negative' by ZN ( $\chi^2 = 32$ ,  $P < 0.001$ ). Thirty-eight 'scanty or 1+' positives were detected by bleach short sedimentation, of which 19 (50%) were declared 'negative' by ZN ( $\chi^2 = 19$ ,  $P = 0.001$ ).

### *Reproducibility assessment*

Table 3 shows the number of AFB-positive smears reported by the two readers, including the number of

**Table 1** Absolute per cent increment gained by the modified techniques compared with conventional ZN microscopy ( $N = 267$ )

	Conventional ZN		Total	Incremental yield $n$ (%)	95%CI
	Positive ( $n = 48$ )	Negative ( $n = 219$ )			
LED fluorescent module					
Bleach centrifugation					
Positive	47	31	78	30 (11)	7.3–15
Negative	1	188	189		
Bleach short sedimentation					
Positive	42	20	62	14 (5)	0.5–8.9
Negative	6	199	205		
Direct auramine smears					
Positive	46	28	74	26 (10)	5.9–13.5
Negative	2	191	193		

ZN = Ziehl-Neelsen; LED = light-emitting diode; CI = confidence interval.

**Table 2** Acid-fast bacilli quantitation results using The Union grading scale and stratified by bacilli density

	Conventional ZN			Total	$P$ value*
	Scanty or 1+ $n$ (%)	2+ or 3+ $n$ (%)	Negative $n$ (%)		
LED fluorescent module					
Bleach centrifugation					
Scanty or 1+	2 (7)	3 (10)	25 (83)	30	<0.0001
2+ or 3+	15 (31)	27 (56)	6 (13)	48	
Short sedimentation					
Scanty or 1+	8 (21)	11 (29)	19 (50)	38	<0.0001
2+ or 3+	9 (38)	15 (63)	0	24	
Direct auramine smears					
Scanty or 1+	3 (12)	1 (4)	22 (85)	26	<0.0001
2+ or 3+	15 (31)	30 (63)	3 (6)	48	

\* Calculated using McNemar's  $\chi^2$ .

LED = light-emitting diode; ZN = Ziehl-Neelsen.

**Table 3** Inter-reader agreement between two slide readers evaluating four microscopy techniques for detection of AFB ( $N = 267$ )

Method evaluated	AFB-positive sputum smears, $n$		Discordant results $n$	$\kappa^*$ (95%CI)
	Reader 1	Reader 2		
Conventional ZN	46	50	10	0.87 (0.79–0.95)
Bleach centrifugation + LED fluorescent module	79	77	10	0.91 (0.86–0.96)
Bleach short sedimentation + LED fluorescent module	61	62	9	0.91 (0.85–0.97)
Direct auramine + LED fluorescent module	76	72	8	0.93 (0.88–0.98)

\*0 = poor; 0.01–0.20 = slight; 0.21–0.40 = fair; 0.41–0.60 = moderate; 0.61–0.80 = substantial; 0.81–1 = almost perfect.

AFB = acid-fast bacilli; CI = confidence interval; ZN = Ziehl-Neelsen; LED = light-emitting diode.

discordant results and  $\kappa$  values for inter-reader variability. The inter-reader agreement was very good ( $\kappa > 0.80$ ) for all of the microscopy techniques evaluated.

#### *Microscopic quality of smears*

Of the 267 smears prepared for each of the four methods, 262 (98%) of the direct auramine slides and 256 (96%) ZN-stained slides were reported as being of good quality. For bleach-treated sputum, 232 (87%) of the smears prepared after centrifugation and 139 (71%) prepared after short sedimentation were reported as being of good quality. Respectively 31 (12%) and 76 (29%) of the smears prepared from bleach-treated sputum following centrifugation and short sedimentation were categorised as thin/unreadable.

## DISCUSSION

Our results showed a significant increase in AFB positivity rate using LED-FM as compared to the conventional ZN method. This finding is in accordance with previous reports.<sup>16–18</sup> Apart from its diagnostic efficiency, LED-FM has several technical and economical benefits.<sup>16,19</sup> These features make LED technology a user-friendly and feasible alternative that can be implemented in resource-poor settings.<sup>8,16,19</sup>

The highest increment was observed by bleach centrifugation, but compared to direct auramine method the difference was not statistically significant. The lack of an appreciable gain from the bleach method might be explained by the fact that under optimal conditions (i.e., appropriate specimen collection and smear preparation), the performance of direct auramine was good.

More than a quarter of the short sedimentation and 12% of the bleach centrifugation smears were regarded as very thin/unreadable. The poor quality of the bleach-treated smears could have contributed to false negative results.<sup>13,20</sup> The risk of false positivity due to transfer of AFB via re-used tubes has also been described.<sup>20</sup> Recently published data have shown lower specificity with no significant increase in sensi-

tivity using LED-FM after bleach sedimentation when compared to direct LED-FM.<sup>21</sup> Compared to direct smear, the preparation of bleach-treated smears took more time and involved extra work. This could potentially hinder its wide implementation in busy laboratories in disease-endemic countries. Another limitation is the instability of bleach solution if stored under suboptimal conditions.<sup>14</sup> Moreover, the bleach method involves centrifuge and conic tubes, which may not be available in resource-poor settings.

There was a relationship between AFB-positive specimens missed by conventional ZN and bacilli density in the modified techniques. More than three quarters of the 'scanty or 1+' positive smears detected by the modified methods were reported as 'negative' by ZN. The relatively high proportion of low-grade positives observed with LED-FM could be attributable to the large TB-HIV (human immunodeficiency virus) co-infection rate in Tanzania, where nearly half of the incident TB cases are co-infected with HIV.<sup>1</sup> It is therefore likely that the benefit of introducing LED technology would be greatest in settings with a high number of paucibacillary TB cases.

In our study, the two slide readers were blinded to each other's results. We observed a very good inter-reader agreement in all methods evaluated. The majority of discordant results reported were from low bacillary load positives. This finding was in agreement with others.<sup>22</sup>

The average reading time taken to evaluate a slide using LED-FM represented 38% of the time required by conventional ZN. The 62% time saving in smear reading could be related to the lower magnification lens used for LED-FM<sup>23</sup> and the quicker scanning of each field as a result of the better visibility of the yellow fluorescent rods.<sup>22</sup> Studies have shown that a significant number of ZN smears are reported as 'negative' due to insufficient examination time;<sup>24,25</sup> the recommended time (5–10 min) for ZN smear evaluation is considerable for busy laboratories.<sup>25</sup> The reduction in examination time observed with LED-FM could therefore be of tremendous benefit for overburdened laboratories in TB-endemic countries.

Another advantage of LED-FM as compared to conventional FM is the possibility of reading slides outside a dark environment.<sup>8,18</sup> In the present study, smear reading was carried out in an ordinary room. LED technology would thus be a suitable option in settings where FM was inaccessible due to the absence of a dark room.

Sputum is viable material for microbiological diagnosis of PTB, and variability in quality may affect the diagnostic yield considerably. Studies have shown that instructing patients in how to produce and submit sputum with good quality improves smear positivity significantly. A randomised study by Khan et al. showed an improvement in TB case detection rates after provision of sputum submission instructions.<sup>9</sup> Another study from Indonesia reported a similar finding.<sup>26</sup> In the present study, the quality of sputum samples was assured by providing appropriate information<sup>9,26</sup> to patients and supervising them while they provided samples.

Our study incorporated features that strengthened its validity. The four duplicate smears were prepared from a single specimen. This approach helped us to control for inter-sample variation. In addition to assessing diagnostic yield, multiple other important outcomes, including average time required for smear reading and test reproducibility, were determined. Finally, the study was strengthened by blinding the slide readers to each other's results to avoid interpretation bias. The study also has certain limitations. First, sensitivity and specificity were not assessed as we did not perform sputum culture, which is the gold standard in TB diagnosis. Second, the study was conducted in a research laboratory with selected samples under controlled conditions. Our results may thus not fully reflect the performance of the methods evaluated in peripheral laboratories. However, our study provides sound evidence that LED FM with direct smear preparation could improve TB diagnosis over the conventional ZN method.

## CONCLUSION

The findings of this study demonstrate the superior diagnostic yield of LED-FM compared with conventional ZN. Importantly, the increased diagnostic yield was more pronounced in low bacilli density specimens. It would therefore be valuable to determine whether LED-based FM is particularly useful in TB-HIV co-infected cases to improve the diagnosis of paucibacillary disease. There was no significant difference in smear positivity in direct and concentrated sputum using LED-FM. Fluorescent LED microscopy with direct smear could help to diagnose PTB rapidly and efficiently. Further studies should ascertain its performance in all-purpose laboratories under routine conditions.

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## R É S U M É

**CONTEXTE :** Hôpital Régional de Mawenzi, au nord de la Tanzanie.

**OBJECTIF :** Déterminer la valeur de la diode émettrice de lumière (LED) lors de l'examen direct des frottis de crachats traités à l'eau de Javel pour le diagnostic de la tuberculose (TB).

**SCHEMA :** Entre décembre 2009 et février 2010, on a rassemblé de manière consécutive les échantillons de crachats provenant de patients suspects de tuberculose pulmonaire qui s'étaient présentés au laboratoire pour un examen des frottis. On a préparé quatre frottis pour chaque échantillon : un frottis coloré conventionnellement par la méthode de Ziehl-Neelsen (ZN), un frottis coloré à l'auramine pour examen direct, un frottis coloré à l'auramine après centrifugation avec eau de Javel et un frottis coloré à l'auramine après une sédimentation de courte durée avec eau de Javel. On a utilisé le microscope à lumière ambiante pour l'examen des frottis colorés au ZN et le microscope à fluorescence LED pour l'examen des frottis colorés à l'auramine.

**RÉSULTATS :** Sur les 267 échantillons de crachats examinés, on en a trouvé comme positifs pour les bacilles acido-résistants (AFB) : 78 (29%) après centrifugation avec eau de Javel, 62 (23%) après sédimentation de courte durée avec eau de Javel, 74 (28%) par examen direct après coloration à l'auramine et 48 (18%) après coloration de ZN. La centrifugation avec eau de Javel a identifié 30 positifs supplémentaires (11%) par rapport à l'examen microscopique par le ZN ( $P < 0,001$ ), mais n'a pas été supérieure à la méthode directe à l'auramine ( $P = 0,46$ ) qui a elle-même trouvé 26 positifs de plus (10%) que l'examen microscopique au ZN ( $P < 0,001$ ). La durée de lecture du frottis a été plus courte avec la LED par fluorescence (1,5 min en moyenne), alors que l'examen microscopique à lumière ambiante exigeait 4 min ( $P < 0,001$ ).

**CONCLUSION :** La LED par fluorescence avec préparation directe du frottis est rapide et efficace. Des études ultérieures sont nécessaires pour confirmer sa performance dans les conditions de routine.

## R E S U M E N

**MARCO DE REFERENCIA:** El Hospital Regional Mawenzi, en el norte de Tanzania.

**OBJETIVO:** Determinar la utilidad de los diodos electroluminiscentes en el estudio de las baciloscopias directas de esputo y las baciloscopias preparadas con lejía, en el diagnóstico de la tuberculosis (TB).

**MÉTODO:** Se recogieron muestras de esputo de los pacientes con presunción clínica de TB pulmonar que acudieron consecutivamente al laboratorio para un examen microscópico del esputo entre diciembre del 2009 y febrero del 2010. De cada muestra se obtuvieron cuatro frotis, que se prepararon mediante tinción convencional de Ziehl-Neelsen (ZN), tinción directa con auramina, centrifugado con lejía y sedimentación corta con lejía con tinción de auramina. Los frotis teñidos con ZN se examinaron en un microscopio de luz y los teñidos con auramina se examinaron en un microscopio de fluorescencia con diodos electroluminiscentes.

**RESULTADOS:** De las 267 muestras de esputo examina-

das hubo 78 baciloscopias positivas (29%) de las examinadas mediante centrifugación con lejía, 62 (23%) de las tratadas mediante sedimentación corta con lejía, 74 (28%) de las examinadas directamente con la tinción de auramina y 48 (11,8%) de los teñidos con ZN. La centrifugación con lejía detectó 30 aislados positivos más (11%) que la microscopia directa con ZN ( $P < 0,001$ ), pero no fue superior al método directo con auramina ( $P = 0,46$ ), el cual ofreció 26 aislados positivos más (10%) que la microscopia con ZN ( $P < 0,001$ ). Con la microscopia fluorescente de diodos electroluminiscentes el tiempo de lectura de los frotis fue más corto (1,5 min en promedio, comparado con 4 min de la microscopia de luz,  $P < 0,001$ ).

**CONCLUSIÓN:** La lectura directa de un frotis con el método fluorescente con diodos electroluminiscentes es rápida y eficaz. Se precisan nuevos estudios con el fin de verificar este rendimiento diagnóstico en las condiciones de la práctica corriente.